Prenatal unhealthy diet, insulin-like growth factor 2 gene (IGF2) methylation, and attention deficit hyperactivity disorder symptoms in youth with early-onset conduct problems

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Background: Conduct problems (CP) and attention deficit hyperactivity disorder (ADHD) are often comorbid and have each been linked to ‘unhealthy diet’. Early-life diet also associates with DNA methylation of the insulin-like growth factor 2 gene (IGF2), involved in fetal and neural development. We investigated the degree to which prenatal high-fat and -sugar diet might relate to ADHD symptoms via IGF2 DNA methylation for early-onset persistent (EOP) versus low CP youth. Methods: Participants were 164 youth with EOP (n = 83) versus low (n = 81) CP drawn from the Avon Longitudinal Study of Parents and Children. We assessed if the interrelationships between high-fat and -sugar diet (prenatal, postnatal), IGF2 methylation (birth and age 7, collected from blood), and ADHD symptoms (age 7–13) differed for EOP versus low CP youth. Results: Prenatal ‘unhealthy diet’ was positively associated with IGF2 methylation at birth for both the EOP and low CP youth. For EOP only: (a) higher IGF2 methylation predicted ADHD symptoms; and (b) prenatal ‘unhealthy diet’ was associated with higher ADHD symptoms indirectly via higher IGF2 methylation. Conclusions: Preventing ‘unhealthy diet’ in pregnancy might reduce the risk of ADHD symptoms in EOP youth via lower offspring IGF2 methylation. Keywords: DNA methylation; Avon Longitudinal Study of Parents and Children; diet; conduct problems; attention deficit hyperactivity disorder; IGF2.

Introduction
Conduct problems (CP) and attention deficit hyperactivity disorder (ADHD) commonly co-occur. Importantly, evidence from family (Faraone, 2000), twin (Thapar, Harrington, & McGuffin, 2001), and molecular genetic (Holmes et al., 2002) studies suggest that that this co-occurrence denotes a more severe, familial, and heritable entity, compared to either CP or ADHD alone. Children with an early-onset and persistent pattern of CP represent a particular at-risk group, as they often show the highest rates of ADHD (Barker, Oliver, & Maughan, 2010), as well as the greatest levels of psychosocial risk exposures in pregnancy (e.g. poverty, maternal anxiety) and the early postnatal years (e.g. harsh parenting, family discordance) (Barker & Maughan, 2009).

One prenatal risk that is a correlate of these psychosocial risks, yet has received far less attention, is diet. ‘Unhealthy diet’ (e.g. high fat/sugar) is of particular interest as it has been reported to associate with both CP and ADHD (Howard et al., 2011; Jacka et al., 2013; Liu, Raine, Venables, & Mednick, 2004; Sonuga-Barke et al., 2013). A potential mechanism that might help explain the link between ‘unhealthy diet’ and CP and ADHD is the epigenetic process of DNA methylation, which is highly responsive to the nutritional environment (Drake et al., 2012), and also associates with CP-related phenotypes (Cecil et al., 2014) and ADHD (Schuch, Utsumi, Costa, Kulikowski, & Muszkat, 2015; van Mil et al., 2014; Walton et al., 2016).

Diet has also been shown to influence methylation of the insulin-like growth factor 2 gene (IGF2) (Heijmans et al., 2008), an imprinted gene that lies close to the insulin and tyrosine hydroxylase genes in a genomic region related to the metabolic regulation of glucose homeostasis, cardiovascular functions, and lipid metabolism (Faience et al., 2010; Ukkola, Sun, & Bouchard, 2001). IGF2 may be of interest to ADHD as it is a major modulator of placental and fetal growth (Constancia et al., 2002) and also plays an integral role in brain development after birth (Pidsley, Dempster, Troakes, Al-Sarraj, & Mill, 2012). Animal and human studies report that IGF2 is associated with developmental abnormalities in the structure and/or function of the cerebellum (Pidsley et al., 2012) and the hippocampus (Chen et al., 2011; Ouchi et al., 2013), both of which are
associated with ADHD (Castellanos et al., 2002; Plessen et al., 2006), as well as other psychiatric disorders such as depression and schizophrenia (Yu, Shen, Zeng, Ma, & Hu, 2013).

Periconceptional risk exposure is associated with abnormal brain development (Jensen et al., 2015), with relevance to CP and ADHD (Fairchild et al., 2011; Rubia, Smith, Brammer, Toone, & Taylor, 2005). Moreover, diet-induced IGF2 DNA methylation modifications occur specifically during the periconceptional period and may persist well in adulthood (Heijmans et al., 2008). Together, these findings suggest that the long-term impact of early-life dietary factors on CP and ADHD may, at least in part, be explained by IGF2 DNA methylation. The current study simultaneously examined, for early-onset persistent (EOP) versus low CP youth, the extent to which unhealthy prenatal and postnatal diet (high fat, high sugar) is associated with ADHD symptoms via DNA methylation of IGF2 (birth and age 7, collected from blood).

Methods

Participants

The Avon Longitudinal Study of Parents and Children (ALSPAC) is a prospective study of children born to 14,541 pregnant women residing in Avon, United Kingdom, with an expected delivery date between April 1, 1991 and December 31, 1992 (85% of eligible population (Fraser et al., 2013)). When compared with 1991 national census data, the ALSPAC sample was found to be similar to the UK population as a whole (Boyd et al., 2013). Ethics approval for the study was obtained from the ALSPAC Law and Ethics Committee as well as Local Research Committees. All participants provided informed consent. The study website contains details of all the data that are available through a fully searchable data dictionary: http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/.

This study uses a subsample (n = 346, 50% male) from a larger study of DNA methylation in ALSPAC, the Accessible Resource for Integrated Epigenomics Studies (ARIES (Reiton et al., 2015), www.ariesepigenomics.org.uk), which follows previously established CP trajectories and has epigenetic data at birth and/or age 7. The CP trajectories, including (a) low CP youth (Barker & Maughan, 2009; Moffitt et al., 2008). Specifically, general mixture modeling was used based on the ‘Conduct Problem’ subscale (4–13 years) of the Strengths and Difficulties Questionnaire (SDQ) (Goodman, 2001). This ‘Epi-genetic Pathways to Conduct Problems Study’ subsample is comparable to the full trajectory sample (n = 7,218) in terms of environmental risk and psychiatric comorbidity (Barker et al., 2010). DNA methylation data were available for 321 youth at birth and 326 at age 7.

In light of the objective of this study, and to ensure a feasible set of statistical analyses, we included youth in the low and early-onset CP trajectories, who had complete data for prenatal ‘unhealthy diet’, IGF2 DNA methylation at birth and ADHD symptoms (n\text{total} = 164; n\text{low} = 81; n\text{EOP} = 83). We did not assess the childhood-limited or adolescent-onset as these youth have different developmental risk pathways than the early-onset and low CP youth (see Barker & Maughan, 2009; Moffitt et al., 2008).

Measures

DNA methylation data. Five hundred nanograms of genomic DNA from cord blood (birth) or peripheral blood (age 7) was bisulfite-converted using the EZ-DNA methylation kit (Zymo Research, Orange, CA). The protocol followed the manufacturer’s instructions using the recommended alternative incubation conditions for use with Illumina Infinium arrays. Illumina HumanMethylation450 BeadChips (Illumina, San Diego, CA) were run following the manufacturer’s protocol with no modifications, and arrays were scanned using an Illumina iScan (software version 3.3.28). Initial quality control of data generated was conducted using Illumina (version 2011.1) to determine the status of staining, extension, hybridization, target removal, bisulfite conversion, specificity, nonpolymorphic, and negative controls. DNA methylation data were only available for samples that passed this stage. Samples were quantile normalized using the dssen function within the waterRmelon package (waterRmelon_1.0.3) (Pidalay et al., 2013) in R and batch-corrected using the ComBat package (Johnson, Li, & Rabinovic, 2007).

We extracted 139 probes that are mapped to IGF2 or overlapping regions adjacent to IGF2, including INS-IGF2 (i.e. position 2150687 to 2183864). For each probe, methylation levels were indexed by beta values (i.e. the ratio of methylated signal relative to the sum of the methylated and unmethylated signals). Factor analysis was used in the total sample to establish the covariance structure among the 139 IGF2 probes in order to extract a smaller set of underlying factors, removing CpGs with low correlations as needed. A three-factor solution showed the best fit to the data. Full details of the factor analysis procedure and results are provided as online supporting information (Appendix S1 and Table S1). We present findings relating specifically to factor 1 (37 probes) because factor 2 (11 probes) and 3 (5 probes) did not correlate with ‘unhealthy diet’ and ADHD symptoms for the EOP and low CP trajectory. See Appendix S2 for the location of the IGF2 methylation probes included in this study, and how these are grouped into factors. See Appendix S3 for the correlations between the IGF2 methylation probes.

High-fat and -sugar diet. The Food Frequency Questionnaire (FFQ; Micali, Northstone, Emmett, Naumann, & Treasure, 2012) was used to assess (a) maternal dietary patterns at 32 weeks of gestation, and (b) what the mother reported feeding to the child at 3, 4.5, and 7 years of age. The FFQ contains a set of questions about the frequency of consumption of a wide variety of food and drink, with higher scores indicating higher frequency of intake. Possible responses were: never or rarely; once in 2 weeks; 1–3 times per week; 4–7 times per week; and more than once daily. Prenatal and postnatal high-fat and -sugar diet scores, indicated by processed food (i.e. fried food, meat pies or pasties, chips) and confectionery (i.e. crisps, chocolate bars, cakes or buns, biscuits) had been previously created using latent factors (Barker, Kirkham, Ng, & Jensen, 2013). For the current analyses, we combined the postnatal diet scores across time (age 3–7) by the use of a latent factor (range factor loadings = .74–.84).

Attention deficit hyperactivity disorder symptoms. Attention deficit hyperactivity disorder (ADHD) symptoms were repeatedly assessed (at age 7, 10, and 13) with the Development and Well-being Assessment (DAWBA); Goodman, Ford, Richards, Gatward, & Meltzer, 2000), a validated semistructured interview. Parents completed open and closed questions about a range of symptoms relevant to youth psychiatric disorders, including ADHD, oppositional defiant disorder (ODD), conduct disorder (CD), generalized anxiety disorder (GAD), and major depressive disorder (MDD). For each
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Model fit was first established using the chi-square statistic. Missing data were handled through full information maximum likelihood. Youth with scores > 3.29 standard deviation from the mean on any study variable were treated as outliers (n = 3) and their scores winsorized (i.e. transformed to match next highest or lowest value).

Results

Descriptive statistics

Table 1 contains the correlations and descriptive statistics of the study variables. These statistics are presented separately for the two CP trajectories. Five results are highlighted. First, in line with previous research, EOP children showed higher levels of ADHD symptoms compared to low CP children. However, means and variances for ADHD differed from zero for the two groups (EOP and low CP youth; all p-values < .001). Second, we found that in EOP but not in low CP youth (a) factor 1 IGF2 mean DNA methylation at birth was positively correlated with ADHD symptoms and (b) factor 1 IGF2 DNA methylation at age 7 was negatively correlated with postnatal cumulative risk. Third, for the EOP youth, ‘unhealthy diet’ correlated at a trend level with IGF2 DNA methylation at birth (r[83] = .20, p = .06) and ADHD (r[83] = .18, p = .10). Fourth, for EOP and low CP youth alike, prenatal cumulative risk was highly correlated with postnatal cumulative risk, but for EOP only, higher postnatal cumulative risk was significantly associated with lower IGF2 DNA methylation at age 7. Fifth, early-onset CP youth significantly differed from the low CP in ODD, GAD, and MDD (p < .05); for EOP and low CP youth alike, prenatal and postnatal JFG2 DNA methylation was not correlated with ODD, GAD, or MDD.

Step 1: Autoregressive cross-lagged (ARCL) model predicting ADHD symptoms

The unadjusted and adjusted models did not differ in terms of significant path coefficients or model comparisons. Therefore, we present the adjusted model only (see Figure S1 for the unadjusted model). Figure 1 depicts the adjusted ARCL model for ‘unhealthy diet’, IGF2 DNA methylation, and youth ADHD symptoms. We tested a series of nested model comparisons, where we assessed differences between EOP and low CP youth in: (a) the auto-regressions; (b) the cross-lagged associations; (c) the prenatal predictions to ADHD symptoms; and (d) the postnatal predictions to ADHD symptoms of ‘unhealthy diet’ and IGF2 DNA methylation. The freely estimated model, which served as the comparison model for all nested tests presented below, showed acceptable fit to the data (χ²[4] = 6.24, p = .18). Because sex differences were not identified across the parameters in EOP versus low CP youth (Δχ²_EOP vs. low = 4.38, p = .88; Δχ²_lower = 9.04, p = .43), we report the results for males and females together.
| Low CP youth Median (interquartile range) | EOP CP youth Median (interquartile range) |
|-----------------------------------------|------------------------------------------|
| -0.04 (0.91) -0.11 (0.85) 0.16 (0.02) 0.17 (0.02) -0.75 a (0.56) -0.63 a (1.03) -0.39 a (0.95) -0.22 a (1.16) -0.17 (0.50) -2.34 a (5.86) 11.1% b |
| 0.09 (0.99) 0.06 (1.45) 0.16 (0.02) 0.17 (0.02) 0.32 (1.20) 0.44 (1.23) -0.09 (0.95) 0.31 (0.73) -0.11 (0.57) 0.32 (7.66) 24.1% b |

EOP, early-onset persistent; CP, conduct problems.

*p-value < .10; *p-value < .05.

Scores were significantly higher for EOP versus low CP youth (p < .05). p-values are derived from Mann–Whitney–Wilcoxon tests.

Measured as frequency (%) of prenatal smoking for EOP (n = 20) versus low CP (n = 9) youth ($\chi^2$ [1] = 3.37, p = .067).
Auto-regressions. The omnibus auto-regressions nested chi-square test constrained two parameters (i.e. stability in diet and IGF2 DNA methylation, respectively). We found temporal stability in diet but not in IGF2 DNA methylation, and these estimates did not significantly vary between EOP and low CP youth ($\Delta \chi^2[2] = 5.96, p = .05$), indicating a similar pattern of auto-regressions across trajectories. This result is statistically significant.

Cross-lagged associations. The omnibus nested chi-square test for the cross-lagged associations constrained the parameters of diet influencing IGF2 DNA methylation and IGF2 DNA methylation influencing diet. Prenatal diet was associated with IGF2 DNA methylation at birth for both EOP and low CP youth, and the strength of this association did not significantly differ between the trajectories ($\Delta \chi^2[3] = 1.98, p = .58$).

Prenatal predictions to ADHD symptoms. The omnibus nested model chi-square difference test constrained the parameters of prenatal diet and IGF2 DNA methylation at age 7 predicting ADHD symptoms. These associations did not significantly vary between EOP and low CP youth ($\Delta \chi^2[2] = 1.70, p = .43$) and were not significant when averaged across all youth.

Step 2: Indirect pathway

For EOP youth, prenatal ‘unhealthy diet’ was indirectly associated with ADHD symptoms via higher IGF2 DNA methylation at birth. The bias-corrected confidence interval (via 10,000 bootstraps) for the indirect pathway of prenatal ‘unhealthy diet’ to ADHD symptoms via IGF2 DNA methylation at birth did not cross zero ($b = .069; 95\% CI .003, .206$). For low CP youth, the 95% CI of this indirect pathway via IGF2 DNA methylation did cross zero ($b = -.015; 95\% CI -.086, .019$). The indirect pathway was different between the EOP and low CP youth: the 95% CI of the difference of the indirect pathways did not cross zero ($b = .084; 95\% CI .224, .005$).

Step 3: Other disorders

In light of the findings above, we repeated the ARCL model to examine the extent to which the association of IGF2 DNA methylation was specific to ADHD symptoms as opposed to other psychiatric disorders (ODD, GAD, and MDD) for EOP versus low CP youth.
For EOP and low CP youth, IGF2 DNA methylation was unrelated to all other disorders (i.e. associations did not survive bootstrapped confidence intervals).

Discussion
In the present study, we used a longitudinal design to investigate prospective associations between ‘unhealthy diet,’ IGF2 DNA methylation, and ADHD symptoms in EOP versus low CP youth. Our results showed that prenatal ‘unhealthy diet’ was positively associated with IGF2 DNA methylation at birth across both EOP and low CP youth. However, only for EOP youth, (a) higher IGF2 DNA methylation at birth predicted ADHD symptoms; and (b) prenatal ‘unhealthy diet’ was associated with higher ADHD symptoms indirectly via higher IGF2 DNA methylation at birth.

The present findings showed a statistical interaction, whereby although DNA methylation levels did not differ between the early-onset and low CP youth, higher levels of DNA methylation were associated with higher symptoms of ADHD for the early-onset but not for the low CP youth. What could be the reason for this? One could posit that the reason could lie in symptoms of ADHD – the same association would manifest for low CP youth if their levels of ADHD were the same as those of the early-onset youth. Another potential explanation could lie in the biological vulnerability of CP with ADHD (Beau-chaine, Hinshaw, & Pang, 2010). Indeed, we identified an indirect effect where higher prenatal intake of unhealthy fats/sugars associated with increased ADHD via higher IGF2 DNA methylation, which may suggest a developmental risk pathway for the early-onset youth alone. It is important to mention that this biological vulnerability may be tapped by other measures of diet (metabolomics) and biology (HPA axis) that are sensitive to stress (Jones, Park, & Ziegler, 2012; Reynolds, Godfrey, Barker, Osmond, & Phillips, 2007).

In this study, we found that for early-onset youth, higher prenatal ‘unhealthy diet’ was correlated with higher IGF2 DNA methylation at birth, but higher postnatal cumulative risk exposure was correlated with lower IGF2 DNA methylation at age 7. Why might DNA methylation associate with the environment differently at birth versus age 7? Findings may reflect two distinct types of risk exposure (i.e. diet vs. cumulative risk), which may differentially influence IGF2 function. On one hand, diet (or prenatal nutrition) has been found to directly affect the metabolic functions of the gene (Ukkola et al., 2001), showing – in the present findings – higher offspring methylation with higher maternal caloric intake, and also lower methylation in the case of maternal caloric deprivation (Dutch Hunger Winter; Heijmans et al., 2008). On the other hand, more distal influences, such as the cumulative risks examined here (e.g. poverty, family discord), may affect IGF2 DNA methylation through stress response pathways, such as increased cortisol activity, which has been found to be associated with lower IGF2 methylation (Vangeel et al., 2015). Given that maternal (or child) stress can co-occur with different dietary patterns (Heijmans et al., 2008), the effects of cortisol and inflammation (Thorburn, Macia, & Mackay, 2014) on IGF2 methylation may be promising avenues for future research.

Several limitations should be considered when interpreting the present results. First, this research is correlational in nature; hence, causality cannot be inferred. However, the present research is based on a longitudinal design, which does allow prospective assessment of prenatal and postnatal effects on DNA methylation and can facilitate the use of methods that can strengthen causal inference (e.g. Mendelian randomization) (Pingault, Cecil, Murray, Munafò, & Viding, 2016; Relton & Smith, 2012). Second, the magnitude of the observed associations was not large, and necessitates replication in larger epidemiological samples. Third, all measures except DNA methylation were based on maternal reports. Hence, the temporal stability of diet may be overestimated. However, it is unlikely that the magnitude of the pathways of interest (i.e. prenatal ‘unhealthy diet’ to IGF2 DNA methylation; IGF2 DNA methylation to ADHD symptoms) is artificially inflated by shared method variance. The use of in-depth interviews for the assessment of ADHD symptoms adds to the robustness of our findings. Fourth, the present study did not identify sex differences in the association between IGF2 methylation and ADHD. Sex differences are nevertheless a promising avenue for future investigation given that boys are generally higher in externalizing problems (such as CP and ADHD). Fifth, the IGF2 locus is a complex genomic region that produces multiple transcripts from alternative promoters, serves different biological functions, and is differentially expressed in different tissues and at different developmental periods. This gene may also shift from monoallelic to biallelic IGF2 promoter methylation during development (Issa, Vertino, Boehm, Newsham, & Baylin, 1996); can show sex differences in monoallelic tissue-specific expression via parent of origin genetic effects (Pidsley et al., 2012); can show loss of imprinting due to diet (Waterland, Lin, Smith, & Jirtle, 2006); and has important functional genomic relationships (Gonzalez-Rodriguez et al., 2016). Therefore, it will be important to establish the extent to which the present results can be replicated and extended with the addition of these molecular and epigenetic mediators and moderators. Finally, it is important to note that at the bivariate level, the association between prenatal ‘unhealthy diet’, IGF2 methylation, and ADHD was not significant (all p ≤ .10), but became significant in the overall autoregressive cross-lag model (i.e. when controlling for all other variables in the model). While such a difference can
Arise when examining bivariate associations (showing the degree of relationship between variables in a pairwise fashion) versus multivariate associations (i.e. which examine a system of predictions; e.g. Tabachnick & Fidell, 2006), the present results should be considered hypothesis-generating and are in need of replication.

In summary, this study is the first to examine IGF2 DNA methylation as a potential intermediary biological mechanism in the association between prenatal diet and ADHD symptoms, for early-onset conduct youth. That we did not find continuity in IGF2 DNA methylation between birth and age 7 may support ideas focusing on the prenatal maternal health as an important risk for postnatal disease vulnerability (Barker, 1995). For example, a prenatal maternal high-fat and -sugar diet may alter the DNA methylation status of the IGF2 gene at birth, which in turn, may increase risk for a range of psychiatric and health disorders. The present study highlights pregnancy as being a promising window of opportunity for reducing the risk of ADHD symptoms associated with the nutritional environment and IGF2 DNA methylation. This is encouraging, given the potentially modifiable nature of nutritional and epigenetic risk factors.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Factor analysis procedure for reducing IGF2 methylation data and results.

Appendix S2. Location of IGF2 methylation probes included in the study.

Appendix S3. Intercorrelations between the IGF2 DNA methylation probes at birth.

Table S1. Confirmatory Factor Model of IGF2 methylation patterns at birth.

Figure S1. Unadjusted model not controlling for prenatal and postnatal cumulative risk factors.

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Key points

- This population-based study used a longitudinal design to investigate, in youth with early-onset persistent (EOP) versus low conduct problems (CP), the interrelations between unhealthy diet and IGF2 DNA methylation in the prediction of attention deficit hyperactivity disorder (ADHD) symptoms.
- Prenatal unhealthy diet was positively associated with IGF2 methylation at birth for both the EOP and low CP youth.
- Only for EOP youth, higher IGF2 methylation predicted ADHD symptoms.
- Only for EOP youth, prenatal unhealthy diet was associated with higher ADHD symptoms indirectly via higher IGF2 methylation.

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